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AWARD NUMBER: DAMD17-03-1-0749

TITLE: Physical Characterization of a Highly Infectious Monodisperse Preparation of TSE Infectivity as a Substrate for Diagnostic Development

PRINCIPAL INVESTIGATOR: Robert G. Rohwer, Ph.D.
Andrew G. Timmes, Ph.D.
Luisa L. Gregori, Ph.D.
Irina Alexeeva, M.D., Ph.D.

CONTRACTING ORGANIZATION: Baltimore Research and Education Foundation, Inc.
Baltimore, Maryland 21201-1524

REPORT DATE: September 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-09-2006	Annual	1 Sep 2005 – 31 Aug 2006

4. TITLE AND SUBTITLE

Physical Characterization of a Highly Infectious Monodisperse Preparation of TSE Infectivity as a Substrate for Diagnostic Development

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0749

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Robert G. Rohwer, Ph.D.; Andrew G. Timmes, Ph.D.
Luisa L. Gregori, Ph.D. and Irina Alexeeva, M.D., Ph.D.

E-Mail: rrohwer@umaryland.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Baltimore Research and Education Foundation, Inc.
Baltimore, Maryland 21201-1524

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Purification and characterization of the infectious agent that causes transmissible spongiform encephalopathies would be a breakthrough in the development of diagnostic tests and treatments for the victims of these fatal neurological diseases. Using a new preparation of highly dispersed, nanofiltered scrapie infected brain homogenate, we have determined the buoyant density and sedimentation constant of PrPres, the only known marker for scrapie infectivity.

Our sucrose step gradient procedure, which combines the ultracentrifugation approaches of equilibrium density and sedimentation rate, succeeded in both purifying and concentrating both PrPres and infectivity. Scaled up production of purified scrapie infectivity involving one liter of dispersed and nanofiltered scrapie homogenate is underway.

We are actively pursuing the original plan of this project, which is to use equilibrium density, sedimentation rate, and other biochemical characteristics to concentrate, purify, and study the scrapie infectious agent. The techniques used are unbiased in their ability to isolate infectious particles, whether they are purely proteinaceous or contain other factors.

15. SUBJECT TERMS
Purification, Centrifugation, Filtration, Diagnostics, Physical Properties

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	22	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction:

Purification and characterization of the infectious agent that causes transmissible spongiform encephalopathies would be a breakthrough in the development of diagnostic tests and treatments for the victims of these fatal neurological diseases. Using a new preparation of highly dispersed, nanofiltered scrapie infected brain homogenate, we have determined the buoyant density and sedimentation constant of PrP^{res}, the only known marker for scrapie infectivity. Three marker particles with known sedimentation constants were used to generate a standard curve for comparison with the behavior of PrP^{res}. We have also used bioassays to determine the buoyant density of infectivity directly and have initiated the bioassays to reveal the sedimentation constant of infectivity. An analysis of the pH stability of PrP^{res} and scrapie infectivity has been completed.

Our sucrose step gradient procedure, which combines the ultracentrifugation approaches of equilibrium density and sedimentation rate, succeeded in both purifying and concentrating both PrP^{res} and infectivity. Scaled up production of purified scrapie infectivity involving one liter of dispersed and nanofiltered scrapie brain homogenate (SBH) is underway. The most abundant contaminant in our purified preparations has been tentatively identified as iron-loaded ferritin, a common contaminant of scrapie preparations. We are actively pursuing the original plan of this project, which is to use equilibrium density, sedimentation rate, and other biochemical characteristics to concentrate, purify, and study the scrapie infectious agent. The techniques used are unbiased in their ability to isolate infectious particles, whether they are purely proteinaceous or contain other factors.

Body:

The Aims referenced below are taken directly from the approved Statement of Work.

Aim I. To measure the sedimentation constant and the buoyant density of the infectivity in the monodisperse preparation and to compare these values to those of PrP^{res} in the preparation. The values obtained for the infectivity will be used to guide the purification procedures in Aim III.
Buoyant density

As projected in our first specific aim, the buoyant density of the infectivity has been determined in a CsCl density gradient equilibrium ultracentrifugation experiment. Two peaks with values of 1.24 +/- 0.01 and 1.29 +/- 0.01 g/ml were identified. These matched the peak densities of PrP^{res}, the only known marker for TSE infectivity. A second CsCl density gradient experiment has been carried out using a new method that produces just one peak of PrP^{res} at 1.29 +/- 0.01 g/ml, but two peaks of infectivity at 1.23 +/- 0.01 and 1.29 +/- 0.01 g/ml (see Figure 1), matching the gradient run in 2004. The ability to create a fraction that is enriched in infectivity relative to PrP^{res} is an exciting development. We intend to replicate this finding and exploit it for increasing the purity of infectivity, either isolating the special form of PrP^{res} that is infectious or finding a scrapie virus.

Knowing the density range of infectivity has already been useful in the development of the step gradient purification protocol described in Aim III.

Sedimentation Constant

We have also developed and carried out a sucrose gradient sedimentation velocity ultracentrifugation experiment that separates particles based on size, mass, density, and shape. We adjusted the parameters so that PrP^{res} would migrate to the middle of the centrifuge tube during the run. Then fractions from a run were inoculated into animals for the bioassay of infectivity. The results of the bioassay should be complete by the end of November. During the long incubation time of the scrapie infection, we have characterized the sedimentation behavior of three marker particles with known sedimentation constants. Two viruses, PhiX174 and PCV1, and one proteinaceous particle, apoferritin, were run in parallel gradients and their locations in the fractions determined, using three different laboratory techniques, see Figure 2. We are thus able to plot a standard curve using data from these characterized markers and plot the experimental particles' locations against the curve, see Figure 2. The PrP^{res} in the inoculated gradient had a heterogeneous sedimentation constant with values in the range of 50-70 Svedbergs (S), which overlapped with the sedimentation constant of PCV1, 52 S. When the bioassay is complete, we will compare the infectivity data to PrP^{res} and the markers.

Aim II. To obtain a monodisperse preparation of TSE infectivity from 10% brain homogenate instead of 1% homogenate, thereby increasing the infectivity of the preparation by a factor of ten.

More Concentrated Preparations

In pursuit of Aim II, we conducted trials with more concentrated brain homogenates to assess their filterability and clarity following filtration. We determined that while a 10% brain homogenate was not efficiently filterable, a 4% preparation was usable. Two liters of 4% brain homogenate were pre-filtered with a 0.2 µm depth filter. Endpoint dilution bioassays have confirmed that no infectivity was lost to the pre-filter, see Table 1. One liter of this filtrate was passaged through two Asahi "20N" nanofilters with 18.4 nm +/- 2 nm pore size. Endpoint dilution bioassays, that are ongoing, have so far shown that two logs of infectivity were lost to the first 18.4 nm filter, but none was lost to the second. This nanofiltration step caused a ten-fold enrichment of infectivity relative to PrP^{res}, because PrP^{res} was reduced three logs by the filter, see Figure 3. We therefore have a liter of a new highly infectious monodisperse preparation with an even smaller particle size than the original monodisperse preparation that came through a 35 nm filter.

We have also produced other infectious stocks of large volume, including two liters of 35 nm filtrate prepared from 1% brain homogenate treated with the nuclease Benzonase and treated twice with the protease trypsin. Two liters of 35 nm filtrate were prepared from 3% brain homogenate treated with Benzonase and trypsin. The above stocks have all been titered by endpoint dilution assay and early indications are that they all contain the high levels of infectivity expected. Other non-titered stocks have also been prepared. Our continuing collaboration with the Asahi company allows us access to their nanofilters, which are uniquely well suited for our purposes.

Nanofiltration of Purified Fibrils

Exploring a different approach to large-scale purification, we used nanofiltration on PrP fibrils prepared in a Diringer-style protocol and subjected to dispersion. Western blots and endpoint dilution titrations revealed that about 6% of the PrPres and infectivity passed through the 35 nm filter. This is close to the same filterability as seen with unpurified brain homogenate. It may be that an efficient way to purify infectivity is to do the fibril prep first and then nanofilter, as opposed to our recent method of putting the entire unpurified brain homogenate through the nanofilter.

Aim III. To purify the monodisperse infectivity using sedimentation to equilibrium, sedimentation velocity, and other methods as necessary including column chromatography and electrophoresis.

Sucrose Step Gradient

Aim III was to purify infectivity using data developed during our studies. Combining data from preliminary density and sedimentation studies, we developed a sucrose step gradient. After carrying out the experiment with 200 ml of monodisperse preparation, we saw that PrP^{res} was concentrated 30-fold. Non-PrP proteins were reduced greater than 100-fold to below the limit of detection by silver stain. We have completed the endpoint dilution titration of the final product of the step gradient and it shows that infectivity was concentrated more than 60-fold over the starting monodisperse preparation, see Table 2.

This successful protocol is now being scaled up for application to one liter of 3% brain 35 nm filtrate, see Figure 4. With this large scale preparation of purified particles, we will be able to take the next logical steps in the characterization of infectivity. The first is to test the theory that PrP is the only component essential for infectivity. We will use acetone precipitation or other methods to concentrate proteins from the purified particles and then analyze the protein constituents by SDS-PAGE, N-terminal sequencing, and mass spectroscopy. We will also use mass spectroscopy and alternative staining techniques to look for other non-protein components such as lipids or polysaccharides as has been reported by others. Finally, we will seek to clone and sequence the residual nucleic acids that are present in every preparation of enriched PrP^{res}. The identification of a TSE-specific nucleic acid would be a breakthrough of enormous importance in the TSE field. The purified particles could also be further purified by divergent methods to remove any as yet undetected contaminants that are not truly part of the infectious agent.

Through a Pilot Project Grant from the Veterans Administration, we have already obtained funds for titration by animal bioassay of the large stock of purified particles that we will prepare. This small grant does not cover any of the costs of production of the stock or its characterization in the laboratory.

Likely identification of Silver Staining Contaminant (SSC)

The purified material from the step gradient procedure contained a substance that was not stainable with coomassie blue or other protein-specific stains, but was detected by silver stain after SDS-PAGE. We now have evidence that this material is iron-loaded ferritin, an oligomeric protein with a hollow sphere ultrastructure, which also stains weakly with coomassie blue. Whereas apoferritin, with no iron stored inside the sphere, can be denatured by boiling in SDS in the presence of reducing agent, iron-loaded ferritin is resistant to denaturation. The large size of the ferritin aggregates causes the protein particles to migrate very slowly in 15% acrylamide, just as SSC does. In an acrylamide gradient gel, however, both ferritin and SSC migrate as a large heterogeneous smear, probably because of variable amounts of iron inside the ferritin spheres, see Figure 5. Like SSC, ferritin is resistant to Proteinase K, even after boiling in the denaturing detergent SDS.

Currently, we are developing a protocol for denaturing ferritin that will be compatible with our infectious samples. Once we can denature SSC into ferritin polypeptides, stainable with anti-ferritin antibodies, we will be certain that SSC has been identified. Next, we will create a protocol for separating ferritin and PrP^{res} and assay the effect on infectivity. As ferritin has contaminated all previous preparations of scrapie infectivity and has been shown to bind PrP^{res} *in vitro*, this experiment may support or put to rest theories about physical links between these two proteinaceous particles.

PH Study

The infectious agent of the transmissible spongiform encephalopathies has been notoriously difficult to purify in a dispersed form and success in such a purification effort may depend on using every available advantage. Many viral purification procedures are limited to gentle conditions that do not inactivate the virions, but the scrapie agent is resistant to extremes of heat and pH. The possibility exists that an elevated pH may improve the efficiency of a future purification step, such as a density gradient or column chromatography, and allow scrapie infectivity to be concentrated in a dispersed form. A large scale bioassay study of the stability of the scrapie agent to increasing alkalinity has been completed, see Table 3. In our preparation of dispersed small particles, originally prepared at pH 7.2, infectivity is stable (or even enhanced) at pH 9 and reduced by one log at pH 10. Alkalinity of pH 11 or higher eliminates infectivity by more than four logs. Interestingly, we observed that 75% of the PrP^{res} in the dispersed preparation became protease sensitive at pH 9 and 99% at pH 10, values that do not match the effect on infectivity, see Figure 6. These results may guide the adjustment of pH during upcoming protocols.

Collaborations

With the help of collaborators at the Rocky Mountain Laboratories and the University of Maryland, we have characterized our purified infectious particles by electron microscopy (EM) (see Figure 7), PrP^{C} *in vitro* conversion assay (see Figure 8), and immunofluorescence with anti-PrP antibodies. The EM revealed thin fibrillar structures, presumably PrPres, and a high concentration of iron-loaded ferritin. Our particles were found to have the same in vitro converting activity as a scrapie brain homogenate of higher PrP concentration, attesting to their quality. Our collaborators are using immunofluorescence to explore the structure of synthetic and natural PrP fibrils. We are currently pursuing the first images of infectious PrP^{sc} by atomic force microscopy.

Aim IV. To measure the filtration pore size limit of the TSE infectivity and of the PrP^{res} amyloid in the monodisperse preparation.

Aim IV has been accomplished in collaboration with the Asahi company. Along with the commercially available 35 nm, 24 nm, 18.4 nm, and 15 nm filters, Asahi constructed special 9 nm +/- 4 nm filters for our use, see Figure 9. Parallel and serial filtrations were carried out which showed, surprisingly, that scrapie infectivity is capable of passing through the smallest size filters. Because PCV1 was blocked by the 9 nm filter, we now know that the most elemental forms of scrapie infectivity are smaller than the smallest known virus.

We would like to further validate the pore size of the 9 nm filters by assaying the filtrates for hamster ferritin, a natural component of brain homogenate. Ferritin is an iron storage protein that assembles into a hollow sphere consisting of 24 protein subunits, surrounding a crystalline iron-oxygen core. The ferritin particle is 13 nm in diameter and theoretically should pass the 15 nm filter, but not the 9 nm filter. If we can demonstrate the absence of ferritin in the 9 nm filtrate, we will be able to support the claim that TSE infectivity can be smaller than 13 nm.

We have three methods available for development that might be sensitive enough to rigorously demonstrate a lack of ferritin in the 9 nm filtrate. The first method we will try is SDS-PAGE followed by silver stain. If this technique lacks sufficient sensitivity, we will develop a western blot using antibodies already in our possession that are specific for mouse or horse ferritin and may cross-react with hamster ferritin. Failing that assay, we will use electron microscopy because the iron core of ferritin is extremely electron dense and easily stands out in a micrograph. We have done preliminary work for all three methods and are confident that one or more will be able to detect the endogenous hamster ferritin in the nanofiltrates.

Sample	$Log_{10}ID_{50}$/ml	ID_{50}/ml
Pre-filter	8.0	9.28×10^7
0.2 µm filtrate	8.3	2.00×10^8
1st 20N filtrate	6.3	2.17×10^6
2nd 20N filtrate	6.4	2.78×10^6

Table 1. Approximately two logs of infectivity are lost by passage of 4% dispersed scrapie brain homogenate through an Asahi Planova 20N (18.4 nm ± 2 nm) filter. No significant change in infectivity was detected after passage through the 0.2 µm pre-filter or a second serial 20N filter. Western blot shows that three logs of PrP^{res} were blocked by the first 20N filter and 0.3 logs by the second. No signal was lost to the pre-filter. The ten-fold difference in loss between titer and PrP^{res} represents a significant enrichment of infectivity.

Sample	$Log_{10}ID_{50}$/ml	ID_{50}/ml
35 nm filtrate	7.0	1.0×10^7
Step Gradient Pool C	8.8	6.32×10^8

Table 2. Infectivity was concentrated 60-fold by the step gradient procedure which also raised the level of PrP^{res} by 30-fold, as measured by quantitative western blot. (The difference in the two values is not statistically significant.) The completion of the bioassay during the last year confirms that infectivity was preserved during the protocol.

Sample	ID_{50}/ml	Log ID_{50}/ml	Titer Change (Log ID_{50}/ml)
pH 7.4 24 hours	8.3×10^6	6.9	-0.1
pH 9.0 24 hours	4.2×10^7	7.6	+0.3
pH 10 24 hours	8.5×10^5	5.9	-1.1
pH 10 24 hours + Benzonase 6 hours	2.2×10^6	6.3	-0.7 (+0.4)
pH 10 24 hours + trypsin 6 hours	1.1×10^6	6.0	-1.0 (+0.1)
pH 11 24 hours	$<5 \times 10^2$	<2.7	- >4.3
pH 12 24 hours	$<5 \times 10^2$	<2.7	- >4.3
1% SBH			
pH 12 24 hours	$<5 \times 10^2$	<2.7	- >5.3

Table 3. Incubation of scrapie infectivity at pH 7.4 led to an insignificant loss of infectivity (compared to previous titrations of the same material, which have yielded an average titer of 7.0 logs ± 0.3 logs). Twenty-four hours at pH 9.0 did not inactivate infectivity, but rather caused an increase that is on the border of statistical significance, 0.3 logs. pH 10 inactivated scrapie infectivity by one log. Further treatment with nuclease or protease caused no further reduction and may have enhanced infectivity in the case of Benzonase Nuclease. (Values in parentheses indicate difference from the pH 10 sample without enzymatic digestion.) The alkalinity of pH 11 and pH 12 destroyed infectivity beyond the limit of detection, or minus 4.3 logs. (Due to the need to dilute the inoculum to reduce toxicity, we are limited to measuring a minimum of 5×10^2 ID_{50}/ml for these samples.) A 1% scrapie brain homogenate sample that started with 8.0 logs of infectivity per ml was inactivated to less than 2.7 logs by incubation at pH 12.

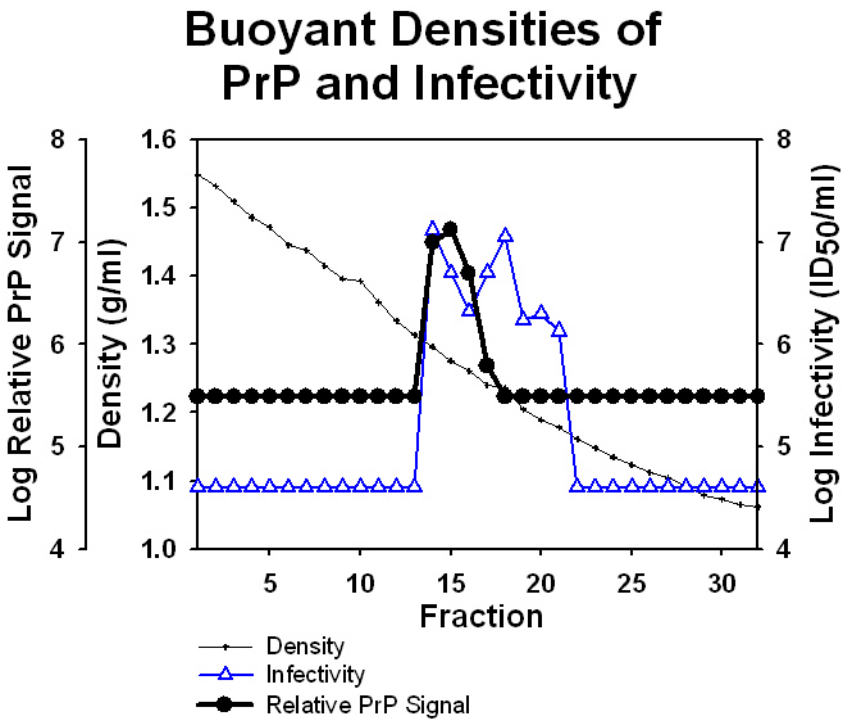

Figure 1. In the new top-loaded CsCl Gradient, PrP^{res} was concentrated into one peak while scrapie infectivity was found in two peaks with a significant shoulder on the lighter peak. 98% of the detectible PrP was in one-tenth of the total gradient. The PrP peak density was 1.28 ± 0.01 g/ml. Infectivity had peak densities of 1.23 and 1.30 ± 0.01 g/ml. The limit of detection for PrP was a 1:43 dilution of the peak signal and all fractions without detectible PrP are plotted at that level. The limit of detection for the incubation time bioassay is 10 ID_{50}/inoculation, which consistently yields infection endpoints before 180 days post inoculation. Considering the dilution of the inoculum and the volume inoculated, our limit of detection was 40,000 ID_{50}/ml. All fractions with less than 10 ID_{50}/inoculation have been plotted at this limit.

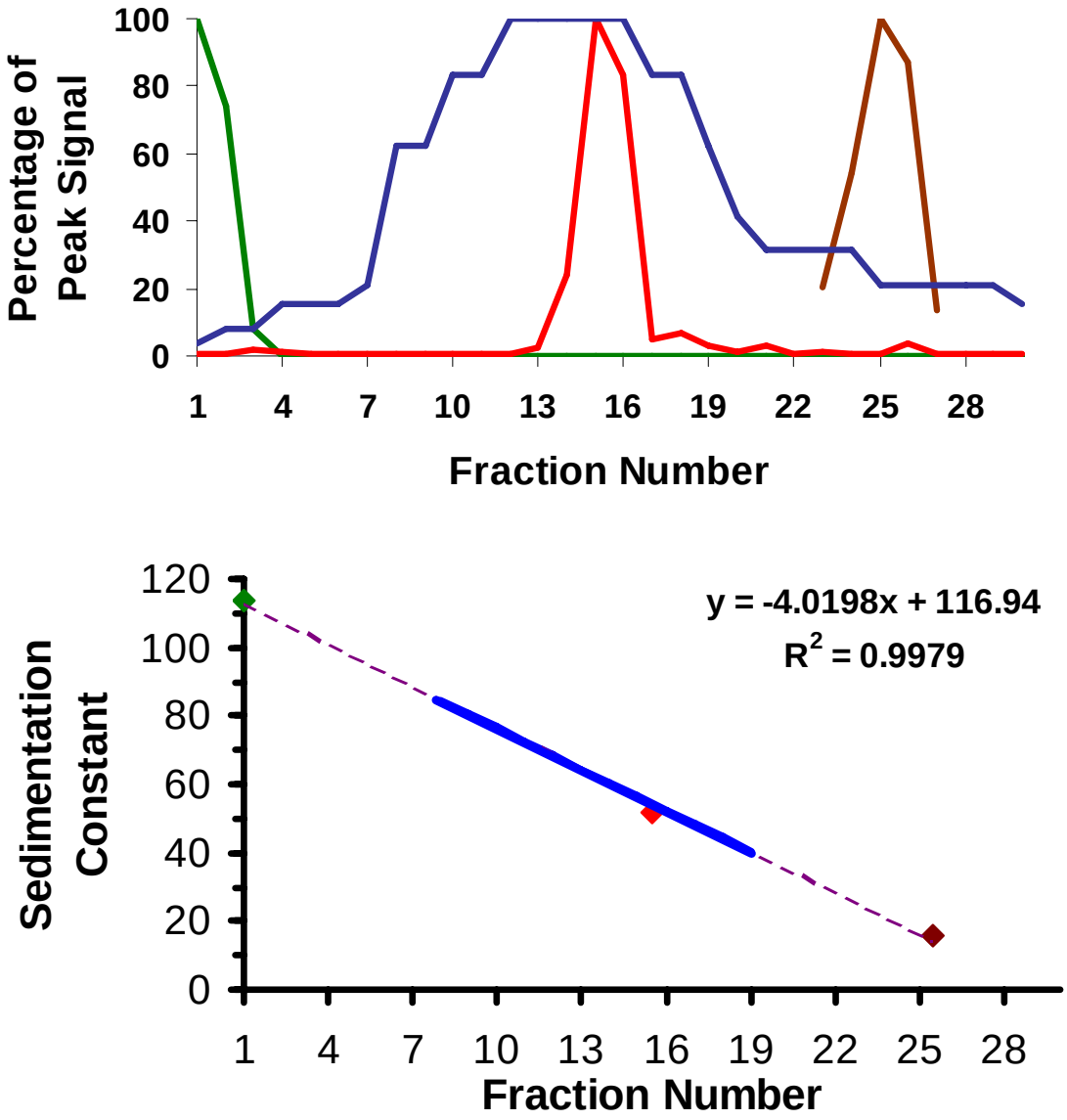

Figure 2. PrP^{res} was predominantly found in fractions 8-19, with a broad peak between fractions 12-16, corresponding to 50 to 70 Svedbergs (S). The marker particles with known constants were found in the following fractions: PhiX174, 114 S, fractions 1 and 2; PCV1, 52 S, fractions 15 and 16; Apoferritin, 18 S, fraction 26. In the top plot, each marker is plotted as a percentage of its own peak signal so that they are all normalized to a peak height of 100%. The bottom figure shows a standard curve generated by plotting the marker particles' positions, which allowed us to interpolate the sedimentation constant range of PrP^{res}. When infectivity data becomes available, it will be added to these plots.

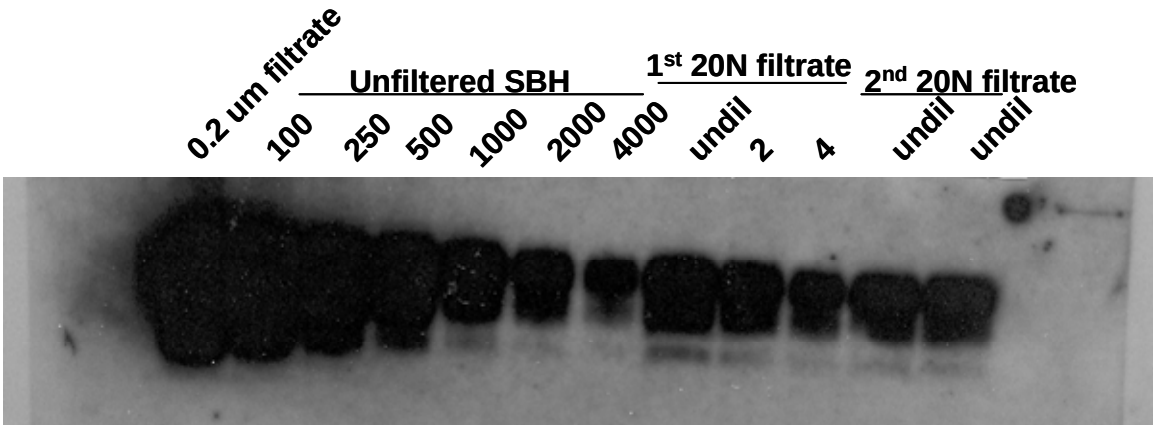

Figure 3. The western blot signal from the second 20N filtrate is equivalent to a 1:2000 dilution of the unfiltered scrapie brain homogenate. The first 20N filtrate has the same signal as a 1:1000 dilution and 0.2 µm filtered material has no loss of signal. The pre-filter thus caused no loss of PrP^{res}, the first 20N filter blocked three logs of PrP^{res}, and the second 20N filter absorbed about half of the remaining PrP^{res} (0.3 log). These data contrast with the titers in Table 1, which show a loss of two logs of infectivity to the first 20N filter and no loss to the second.

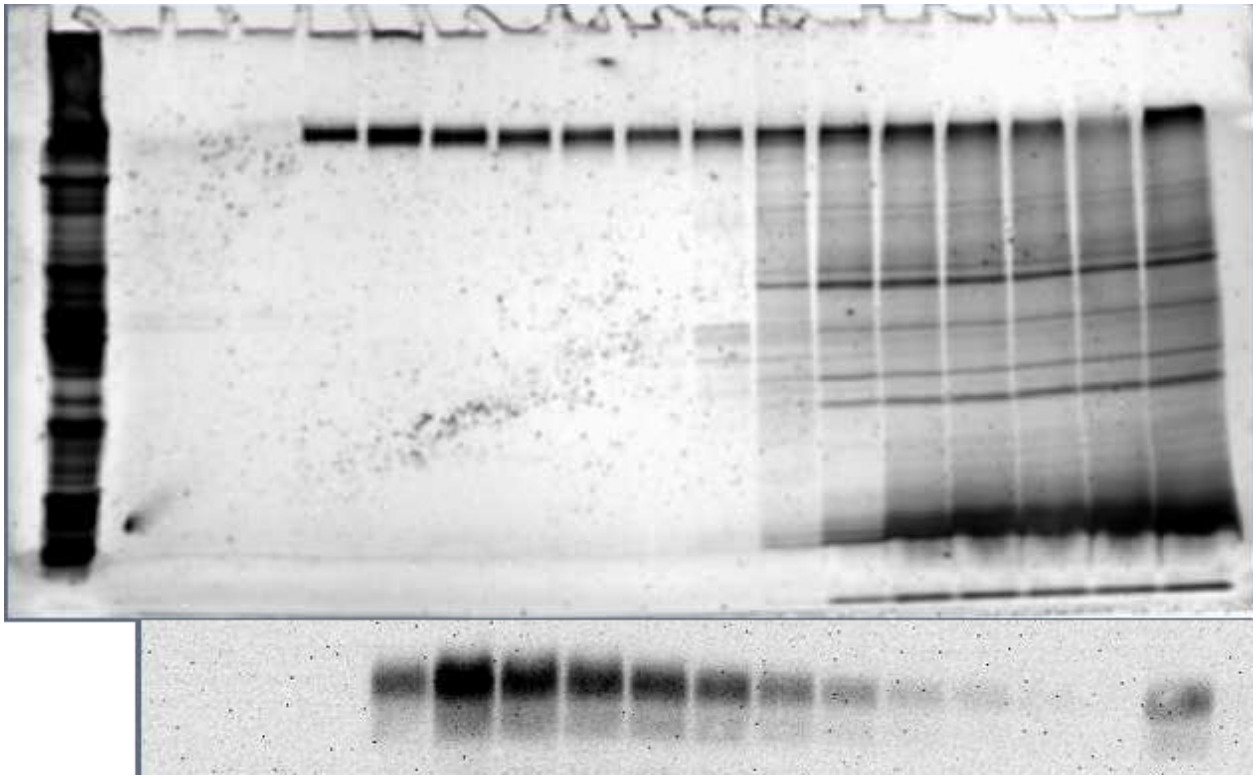

Figure 4. Fractions from the scaled up sucrose step gradient were examined by BIO-RAD Silver Stain Plus kit (top image) and western blot with 3F4 antibody (bottom image). Left to right: Broad Range MW Std, Fractions 1-15, Combined fractions 16-30, Unspun starting material (3% SBH 35 nm filtrate treated with Benzonase and trypsin). Sample began the run at the location of fractions 14 to 30. During the run, PrP^{res} sedimented into the sucrose layers as far as fraction 5, while soluble protein only penetrated as far as fraction 10. The silver stained top image reveals that the silver staining contaminant, possibly ferritin, sedimented farther into the dense sucrose cushion than PrP^{res}. Lane 17 shows that SSC is depleted from the top half of the centrifuge tube (because it all sedimented into the bottom half).

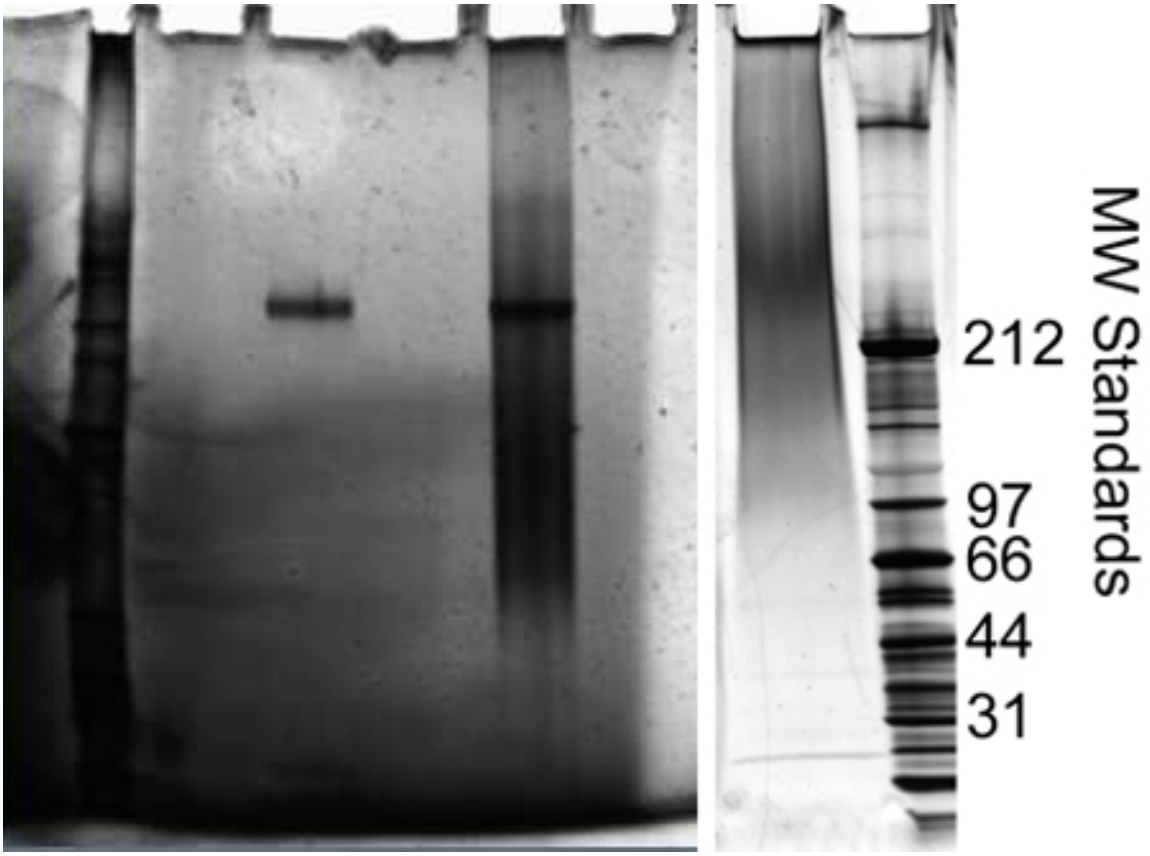

Figure 5. The silver staining contaminant (SSC), lane 1 right image, appears as a smear of heterogeneous species in SDS-PAGE with a 4-15% gradient gel and silver stain. Ferritin with iron, lane 5 left image, also migrates as a smear, perhaps due to variable iron content in the particles. Apoferritin, without iron, lane 3 left image, migrates as a single species. The MW standards, lane 1 left image and lane 2 right image, might give the appearance that the contaminant has an average molecular weight higher than myosin (212 kDa), but such a comparison is meaningless if SSC is non-denatured ferritin protein. Migration in SDS-PAGE is affected by charge, binding to SDS, and the shape of a macromolecule in addition to molecular weight. Ferritin will not have the same ratio of detergent to protein as denatured proteins and will remain in a spherical shape whereas denatured proteins are elongated.

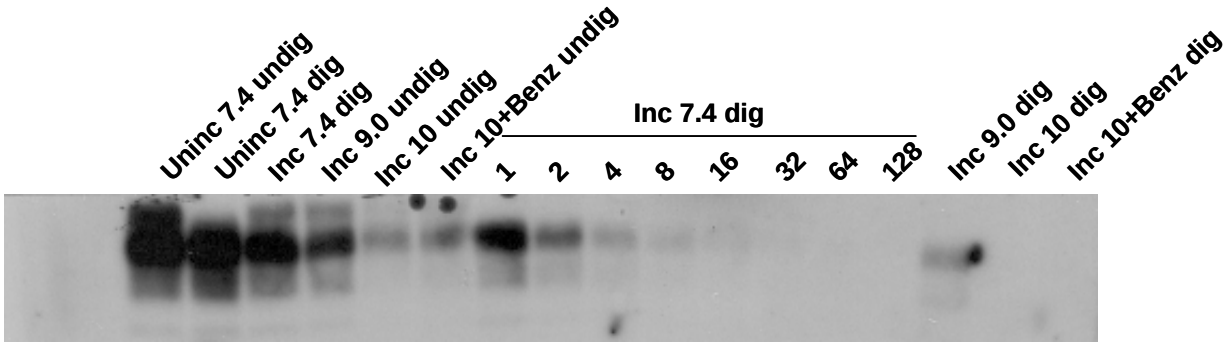

Figure 6. 35 nm filtrate of dispersed scrapie brain homogenate was incubated (inc) at different pH values for 24 hours and then digested (dig) with trypsin. Samples were neutralized before running in SDS-PAGE for western blot. (Non-neutralized samples showed reduced western blot signal.) Unincubated (uninc) samples contain full-length PrP^{C} and PrP^{res}. Digested samples contain only truncated PrP^{res}. Sample that was incubated at pH 9.0 and then digested has a signal equivalent to a 1:4 digestion of sample incubated at 7.4 and digested. The effect of pH 9.0 was to render 75% of the PrP^{res} protease-sensitive. The amount of protease-resistant PrP^{res} remaining after incubation at pH 10 is below the limit of detection in this experiment.

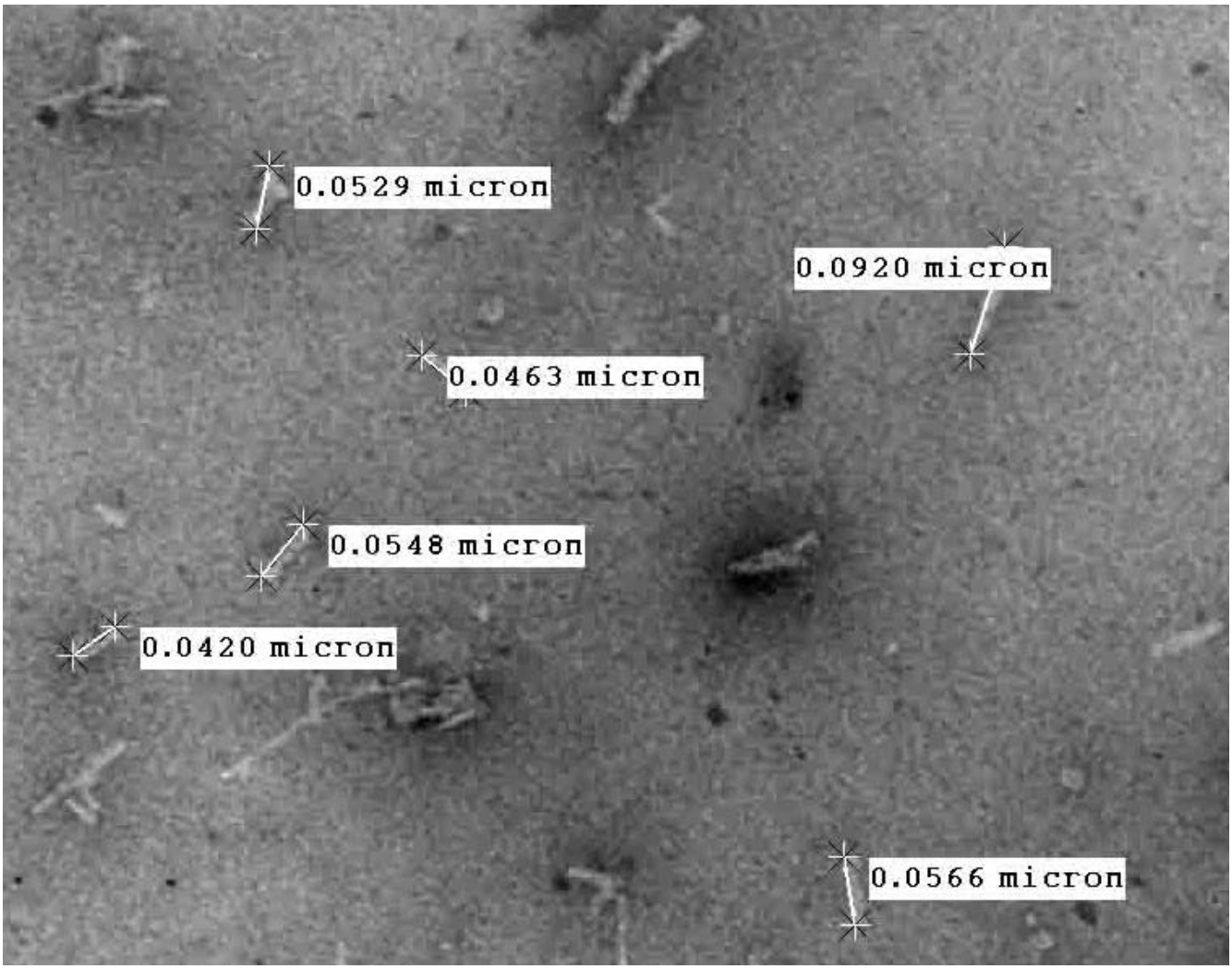

Figure 7. Our collaborator at the Rocky Mountain Laboratories, NIAID, NIH provided this electron micrograph of our step gradient pool. The measurements refer to lengths of fibrils that are presumably PrP^{res}. Such small fibrils were not present in a control step gradient pool prepared from normal brain homogenate. Some fibrils appear to be singular and others pairs or higher order aggregates. Small black dots are the iron cores of ferritin particles.

Cell-free conversion (No Gdn-HCl pretreatment)

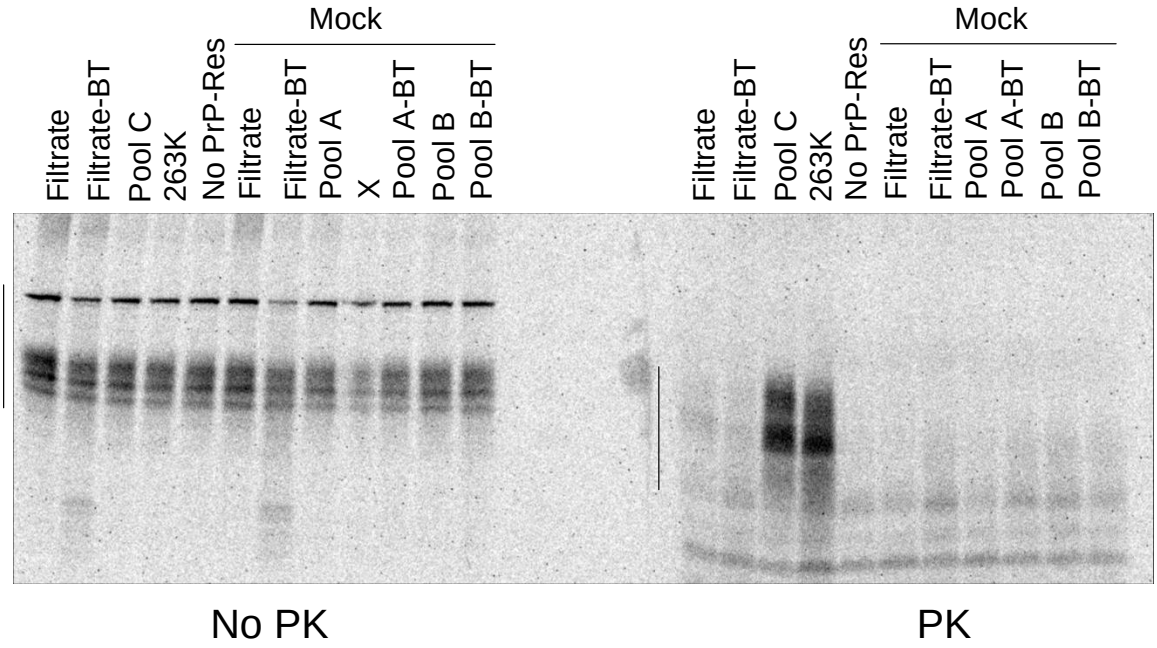

Figure 8. Another collaborator at the Rocky Mountain Laboratories, NIAID, NIH performed a cell-free conversion assay on our step gradient pool, called Pool C in the figure. 100ng/reaction of purified 263K PrP^{res} or 2.5µl/reaction of each fraction was incubated with ~1ng of 35S-radiolabeled PrP^{sen} for 2 days at 37°C. Non-converted PrPsen remains protease sensitive and is removed by digestion with Proteinase K (PK). Our purified particles had the same converting activity as a 10% scrapie brain homogenate (263K in the figure). Mock reactions using samples prepared from normal brain had no activity.

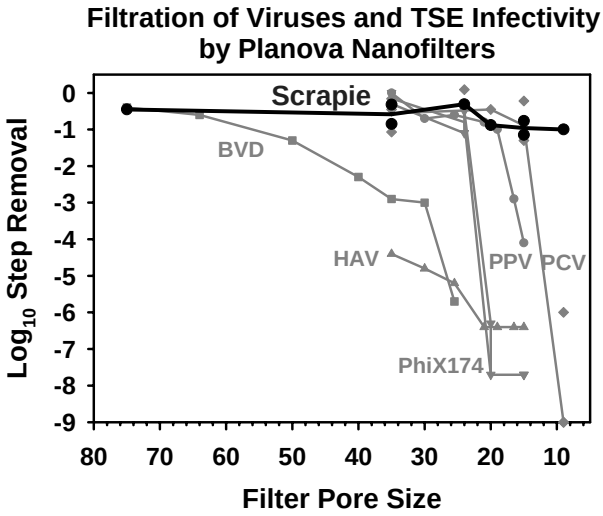

Figure 9. When dispersed scrapie brain homogenate was passed through an Asahi 75 nm filter, there was no retention of infectivity. Moreover, the 75 nm filtrate passed through a 35 nm filter with negligible loss of infectivity indicating a particle size smaller than 35 nm. The 35 nm filtrate in turn passed, with only non-specific losses, through 24 nm, 18.4 nm, and 15 nm filters. A PhiX174 phage control passed the 24 nm filter but was completely blocked by the 18.4 nm filter consistent with its 24 nm diameter. A porcine circovirus (PCV) control passed all filters with an efficiency comparable to the scrapie infectivity. PCV is the smallest known virus of which we are aware. Subsequent passage through a nominal 9 nm filter custom built for us by Asahi completely blocked PCV but continued to pass TSE infectivity. This shows that the size of the elemental particle is smaller than the smallest known virus, PCV, with a 17 nm diameter as measured by electron microscopy. We have not yet been able to validate the pore size of the nominal 9 nm filter, but hope that examinations of the ferritin content of the filtrates will accomplish this goal. Ferritin, an endogenous component of hamster brain homogenate, has a diameter of 13 nm.

Key Research Accomplishments:

The Aims referenced below are taken directly from the approved Statement of Work.

Aim I - To measure the sedimentation constant and the buoyant density of the infectivity in the monodisperse preparation and to compare these values to those of PrP^{res} in the preparation. The values obtained for the infectivity will be used to guide the purification procedures in Aim III.

- Sedimentation Constant of PrP^{res} determined (infectivity measurement underway)
- Buoyant Density of PrP^{res} and infectivity measured

Aim II. To obtain a monodisperse preparation of TSE infectivity from 10% brain homogenate instead of 1% homogenate, thereby increasing the infectivity of the preparation by a factor of ten.

- Brain homogenate and dispersant concentrations for dispersed preparations were optimized
- Six liters of filtered and nanofiltered preparations have been produced and titrated by animal bioassay

Aim III. To purify the monodisperse infectivity using sedimentation to equilibrium, sedimentation velocity, and other methods as necessary including column chromatography and electrophoresis.

- Optimized Sucrose Step Gradient concentrated PrP^{res} and infectivity
- Scaled up Sucrose Step Gradient is underway; separate funding for the animal bioassay has been acquired
- Likely identified Silver Staining Contaminant as ferritin with iron
- Productive collaborations have provided information about our purified infectious particles
- Study of pH stability of PrP^{res} and infectivity completed

Aim IV. To measure the filtration pore size limit of the TSE infectivity and of the PrP^{res} amyloid in the monodisperse preparation.

- Nanofiltration down to a putative 9 nm pore size has been carried out and completed bioassays of infectivity have shown that some portion of the infectivity is able to pass through the smallest filters
- Identified ferritin as a validation tool for the 9 nm filters

Conclusions:

We have developed a protocol that purifies PrP^{res} from our nanofiltrate of dispersed scrapie brain homogenate and verified by bioassay that infectivity is also concentrated. We have scaled up the procedure to process one liter of nanofiltrate and create a large amount of purified particles. We believe that this large amount is necessary to examine the possible non-PrP components of infectivity that may be present at low concentrations.

Through our characterization of the physical properties of PrP^{res} and infectivity, we have identified three potential pathways for enriching infectivity relative to PrP^{res}. These are incubation at pH 9.0, which renders 75% of PrP^{res} protease sensitive, ultracentrifugation in a CsCl gradient, which produces a band of high infectivity and low PrP^{res}, and nanofiltration. A preparation with higher specific infectivity relative to PrP^{res} may enable the identification of the special form of PrP^{res} that is most infectious or non-PrP components.

With the extension of the term of the grant NP020079 (without increasing the amount of the award) and the revision of the statement of work, we will be able to carry forward the momentum we have achieved in defining the physical properties and molecular constituents of TSE infectivity.

REVISED STATEMENT OF WORK FOR CDMRP GRANT NP020079

I. To prepare a large stock of purified infectious particles from one liter of infected brain homogenate.
II. To characterize the molecular components of the purified infectivity including proteins, lipids, polysaccharides, and nucleic acids.
III. To validate the extremely small filtration pore size of the TSE infectivity by assaying ferritin levels in the nanofiltrates of monodisperse preparation.